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*Original scientific paper*

## **PROTEINS IN SEED AND SEEDLINGS OF SELECTED AUSTRIAN PINE (*Pinus nigra Arnold*) TREES AS GENETIC MARKERS TOLERANT TO DROUGHT**

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A precondition necessary for creation and selection of genotypes  
tolerant of stress conditions is a study of physiological, biochemical and  
molecular bases of their adaptive reaction to stress. The study includes 40  
lines of free pollination originating from 5 provenances: Sutjeska,  
Višegrad, Tara, Teslic and Durmitor (B&H, Serbia and Monetenegro). Two  
populations were selected from each provenance, i.e.: 5 lines of free  
pollination represent the population growing on the cliffs and 3 lines of  
free pollination represent the population growing at the best site of  
Austrian pine. Specific characteristics of the studied provenances,

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populations, and free pollination lines were confirmed by the analyses in the salt-soluble proteins. The identical protein composition was proved in a small number of cases in the replicates of the same free pollination line, which indicates a high intra-line variability, which can be the result of the effect of father, as well as of heterozygosity of mother trees. The analyses of protein composition of seed showed considerable differences at provenance level. Inter-line, population and provenance differences, and also, the interaction between the origin and drought factor, were recorded for 9-day-old seedlings germinated in induced drought conditions and in normal conditions.

*Key words:* Austrian pine, half sib lines, proteins, seed, seedlings

## INTRODUCTION

Basic characteristics of genetic markers are neutral phenotype expression, codominancy and absence of epistatic interaction between the loci, which are used as markers. Molecular markers are mainly used to identify, localize and characterize genes, as well to check genetic control of quantitative characteristics, yield, resistance to diseases and stressful conditions (MLADENović DRINIĆ, S., 1995).

Protein analysis is a basic biochemical procedure to determine differences between certain trees and populations, also. If we know the genetic quality of planting material and the reaction to environment factors, we have a base to develop a strategy for successful growing and management of forest cultures. Moreover, if we have the knowledge on the reaction of physiological processes and limiting environmental factors by applying genetic and selective programmes we can produce the planting material of desired and defined characteristics. In that way the produced planting material would withstand stressful environmental factors, thanks to its morphological, anatomical and physiological characteristics. Therefore, molecular markers can help us to understand the potential, better efficiency and sustainable use of biodiversity. Apart from morphological and cytogenetic markers, protein markers which are frequently used are polymorph molecular markers at gene product level (KONSTANTINOV, K., et. al. 2000). There are few reasons to use protein markers in seed: content of protein in seed is high and stable, seed represents a well defined pheno-phase in plant development, it demands rather small space for long term conservation of seed vitality of larger number of gene types, tissue is relatively clear (absence of tannin, phenol, etc.).

When we use molecular markers to determine presence/absence of differences between analyzed individuals/populations, one of the approach which can help study molecular mechanisms in stress resistance is testing gene expression in stressful conditions in order to identify genes through their products- RNK

molecules and proteins, which have adaptive significance (KOVAČEVIĆ, D, et al. 1995).

There were many studies on protein structure in different tissues, which applied these markers as molecular marks, among them we can mention a few studies which focused their activities on the analysis of *Pinus* genus protein marker (COSTA, P., ET AL., 1998; 1999; 1999; 2000; GERBER S, et al., 1993; 1998; NIKOLIĆ, Đ., 1982; PLOMION, C., et al. 1995; 1997). Many research activities were carried out on Austrian pine with the help of morphometric parameters, and therefore many papers have been published (ISAJEV, V., et al., 1994; NIKOLIĆ, Đ., 1982; VLAINIĆ, A., 1993; MATARUGA, M., 1997; 1998; 2003; VIDAKOVIĆ, M., 1957; 1960; 1974; VIDAKOVIĆ, M., et al., 1976).

#### OBJECT OF THE RESEARCH WORK AND APPLIED METHODS

Protein complex analysis in seed and seedlings, applying WANG'S et. al. (1994) method, was used in order to show the index of tested material genetic specific quality. Research work included 40 free pollination lines originating from 5 provenances: Sutjeska, Višegrad, Tara, Teslić and Durmitor. Two populations were selected out from each provenance: 5 free pollination lines representing population growing on extremely difficult habitat (fig. 1) and 3 free pollination lines representing population growing on the best Austrian pine habitat (fig 2).

Protein extraction was done from haploid megagametophyte and diploid germ tissue for all 40 free pollination lines. Total number of seed was 1200, with 10 seeds for one repetition (3 repetitions x 10 seeds = 30 seeds per line). Proteins were separated by their molecular mass by electrophoresis on polyacrylamide gel. Qualitative analysis included 19 polypeptides of different molecular mass and identified the genotype through polypeptide presence or absence (fig 3). All laboratory activities were performed in biotechnology and physiology laboratories of the Maize Research Institute Zemun Polje.

Protein analysis of 9-days-old seedlings germinated in distilled water and 4% sucrose solution was done, beside the protein analysis in free pollination line seeds. The goal of this research work was to monitor changes at protein level, as an answer to water deficit in terms of seed germination, i.e. protein polymorphism of different lines depending on their origin. Protein analysis of seedlings was done on samples from three provenances (Višegrad, Teslić and Durmitor). These provenances are singled out as they come from the remotest part, those are provenances with largest differences vertical and horizontal extension, as well the provenances with populations of the highest and lowest differences considering habitat conditions.

First sample on the gel, which was used to analyze protein complex, is a protein marker of known molecular mass. It is followed by 24 samples where first three present line 1 from that provenance in three repetitions all to the eighth line (8 lines x 3 repetitions=24). Marker of a known molecular mass is on the gel, which was used to analyze protein complex in seedlings. It is followed by 16

samples, 8 of them are half sib progeny, where the first strip presents protein complex from seedling in distilled water, and the second one is of a germ in 4% sucrose solution (8 lines x seedlings in distilled water and seedlings in sucrose=16).



Figure 1. Austrian pine growing on cliffs



Figure 2. Austrian pine growing on the most productive habitat

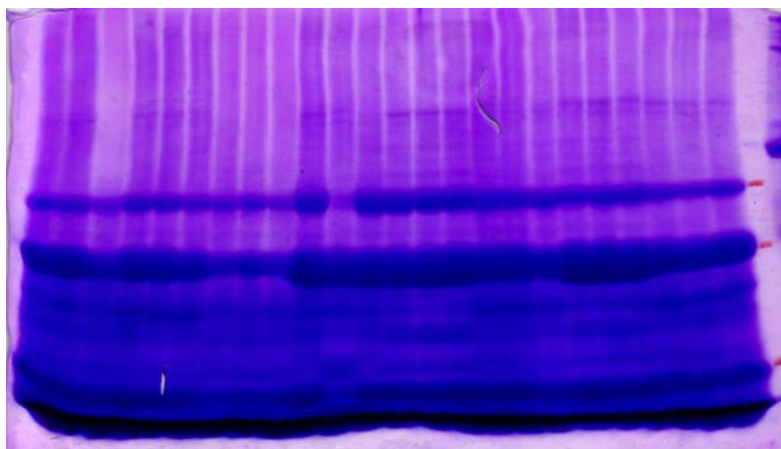


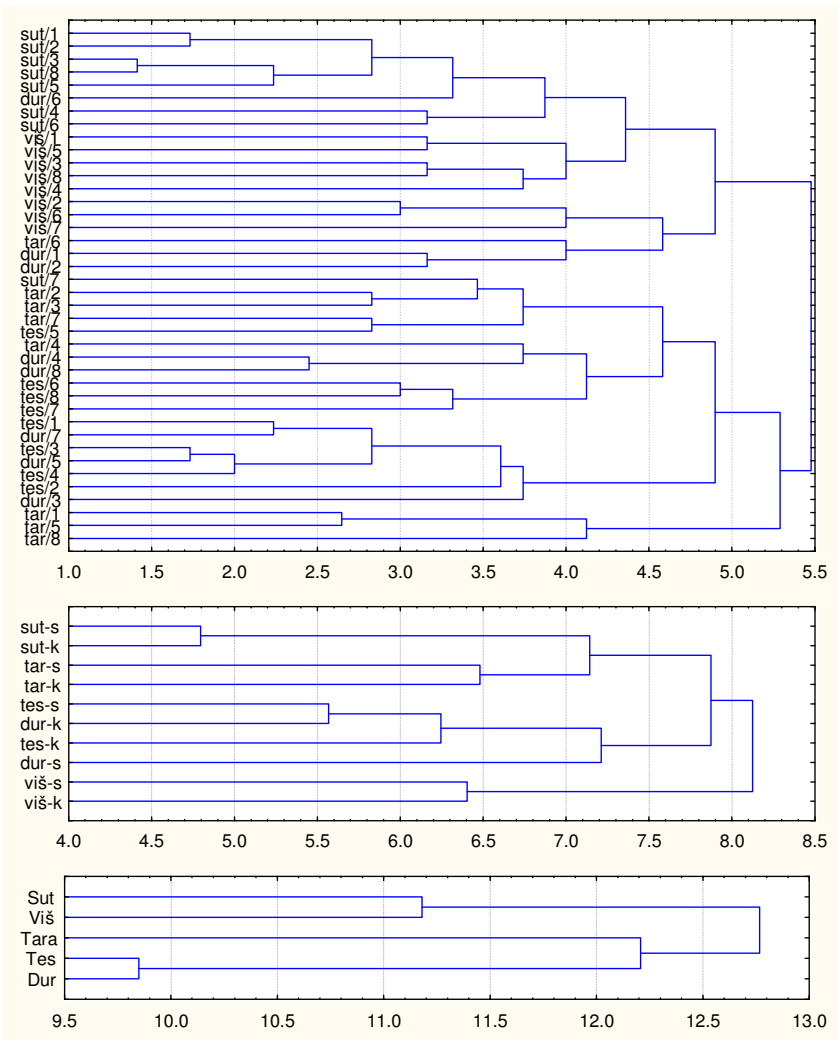
Figure 3. Electrophoresis gel for the seed of 8 test trees in 3 repetitons

## RESULTS

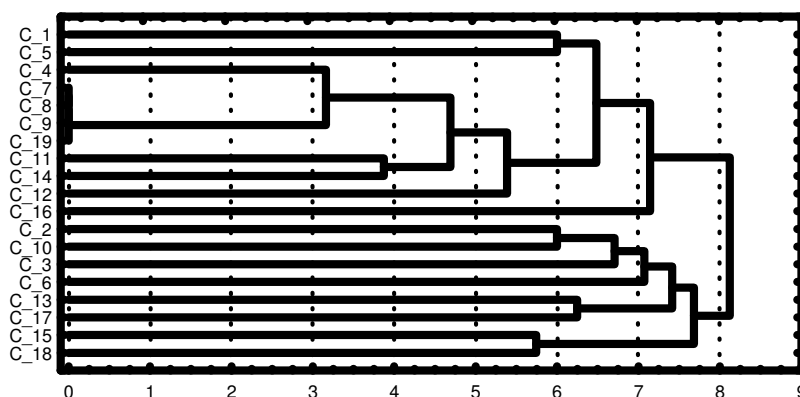
Electrophoregram analysis of protein complex in seed identified the highest differences between provenances (graph. 1). These results confirm formerly obtained results that the habitat does not influence protein complex (FORREST, G., 1994), as well the results showing variability between the analyzed populations, which were spatially isolated (BAHRMAN, N., et al., 1994; 1997). This is the reason why the polypeptide, which could be brought into the correlation with differences of habitat condition (populations) where the seed originated from, had not been identified.

Analysis of polypeptide arrangement, for each line individually, we can notice by using cluster analysis dendrogram (graph 1) that lines grouped into two groups (with a slight deviation). The first group is mostly made of lines from Sutjeska and Višegrad provenances (in geographical sense, they are relatively in vicinity), and the other group is made of lines from the other three provenances. Lines originating from Teslić and Durmitor provenances group without any rule, unlike the lines originating from Tara group which within this group as two entireties. There is one exception in the above mentioned regular grouping, it is line sutjeska/7 which showed higher similarity to lines of Tara provenance and line durmitor/6, which grouped with Sutjeska provenance lines at lower distance. As it was earlier mentioned, we did not notice any grouping which depended on habitat conditions where the tree grew. At population level, the populations group within individual provenances at the lowest distances. In this way they, once more confirm the insignificant difference in habitat conditions and differences between populations, and emphasize the importance of provenances (graph 1.). Although Teslić and Durmitor provenances differ in spatial point of view (in vertical and horizontal sense), at provenance level they group at the lowest distance as the most similar provenances. The later research work of 158 analyzed morphological and anatomical characteristics of these test trees, seed and seedlings at half sib line level, confirmed this type of grouping.

In order to define polypeptide variability (steadybility) in all 40 free pollination lines, proteins are marked from 1 to 19 (at seeds), and 1 to 22 (at seedlings) beginning with proteins having the highest values kD (kiloDaltons) to proteins having the lowest values i.e. at electrophoregrams from up to down. In this way marked polypeptides cluster analysis was done at seed level (graph 2). Here we can single out proteins which can be defined as stable - monomorph, i.e they occur in all tested lines and repetitions disregarding habitat conditions they originate from (7, 8, 9, 19). After this group we can single out proteins which are marked as 1, 4, 5, 7, 8, 9, 11, 12, 14, 16 and 19, present in the most of the lines, while the proteins marked with 2, 3, 6, 10, 13, 15, 17, 18, are very unsteady, i.e. they rarely occur in lines, repetitions or provenances - polymorph.



Graph 1.Cluster analysis of lines on the basis of the content of salt soluble proteins in seed in regard to half sib lines, populations and provenances



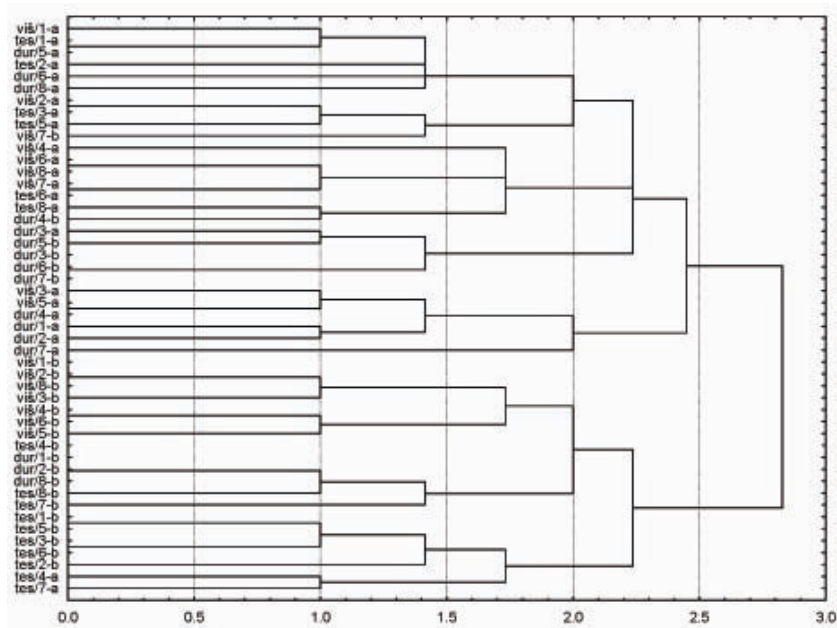
Graph 2. Cluster analysis of protein stripes on the basis of the content of salt soluble proteins in seed

Analysis of proteins isolated from 9-days-old seedlings germinated in water and osmoticum - 5% of sucrose solution was done in order to define gene expression in stressful conditions.

Cluster analysis, according to a protein electrophoregram, (graf. 3.) clearly separates two homogenous groups, which depend on the substrate seed germinated on (distilled water or 4% sucrose solution). Significant deviations have been noticed with seedlings in distilled water line tes/4 and 7, which have according to protein complex been grouped in seedlings in sucrose group. Seedlings germinated in drought conditions, lines viš/7; dur/3,4,5,6,7, have by their protein structure been grouped in seedlings in normal conditions group (graf 3). At population level, the process of grouping by provenances, where lines originate from, is almost regular (dur/4 is exceptiona), while at population level populations from Višegrad provenance group at higher distance (graph 4.). Populations from Teslić and Durmitor provenances grouped at lower distances, indicating inter-population differences at protein level for seedlings germinated in water and 4% sucrose solution. Obtained results confirm significant differences in populations from Višegrad provenance, which differences occurred even later when exposed to direct drought effect.

This speaks in favour of the earlier obtained results at the individual polypeptide level. They showed that gene expression, as the consequence of different germination conditions, depends on population, i.e. habitat differences. Protein marked as no. 1, which mother trees grow on rocks, does not occur in drought conditions. This is the case with all half sib lines. Nevertheless, it occurs in check population (lines tes/7,8 and viš/7). In Višegrad provenance, protein no. 18 can be defines as stable in lines growing on rocks (regardless of germination

conditions). In the lines of check populations this protein occurs only drought conditions (graph 4).

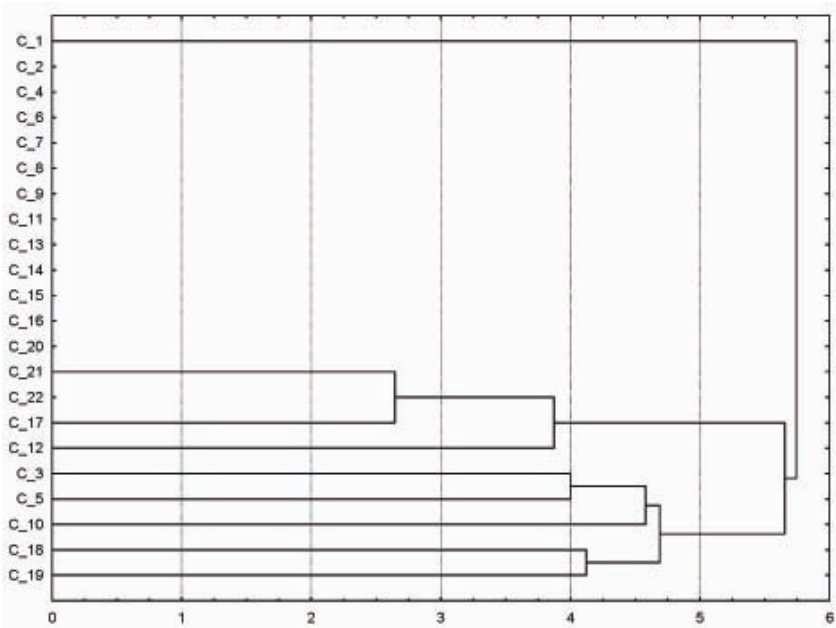


Graph 3. Cluster analysis of protein stripes on the basis of the content of salt soluble proteins in 9-days-old seedlings (a- in distilled water; b- in 4% sucrose solution)

Results obtained in polypeptide analysis show the presence of:

- polypeptides which are regardless of germination conditions always stable (polypeptides marked as 2,4,6,7,8,9,11,13,14,15,16,20,21,22);
- polypeptides which almost always occur in seedlings grown from seed and exposed to drought conditions (polypeptides marked as 3, 5, 10, 19);
- polypeptides which presence/absence can not be connected with the conditions seedlings germinated in (water or sucrose), (polypeptide marked as 1);
- polypeptides which in a few cases occur in seedlings grown from seed and exposed to drought conditions (polypeptides marked as no. 17-viš/3,4,5; dur/1,2,4,7; protein 18-viš/6,7,8; );
- polypeptides which in a few cases do not occur in seedlings grown from seed and exposed to drought conditions (polypeptides no. 12-viš/1,2,3,4,5,6,8).

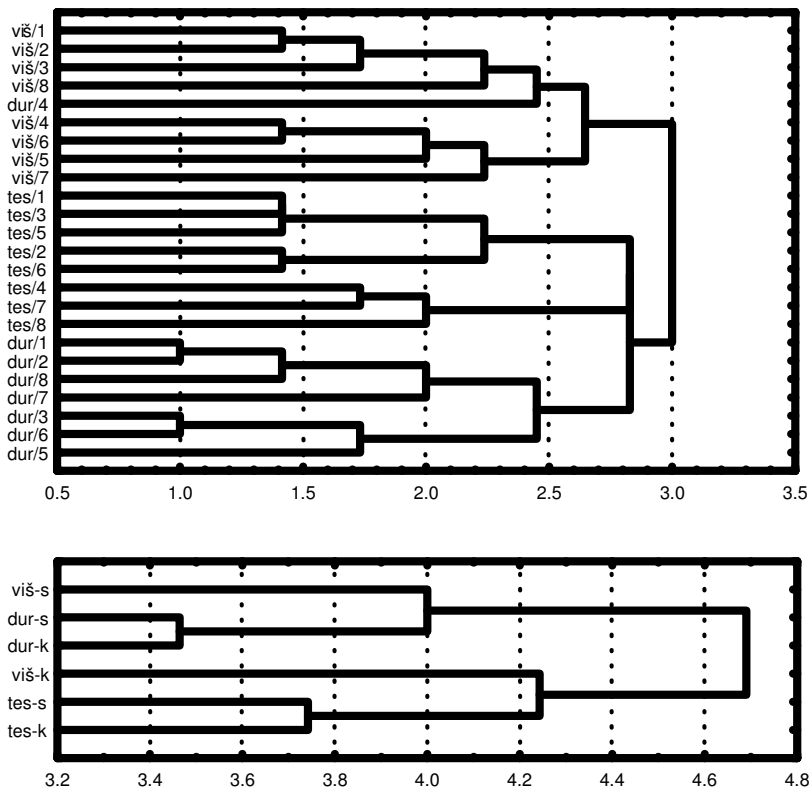




Graph 4. Cluster analysis of 22 protein stripes on the basis of the content of salt soluble proteins in 9-days-old seedlings

These results identified that polypeptides' presence/absence regardless of the germination conditions, as well it identified the group of polypeptides which presence/absence depends on germination period and free line pollination reaction to current conditions.

Depending on habitat conditions seed germinates from, the protein complex in seed can be defines as stable, i.e. differences are attributed to inter-provenance differences. However, ots analysis at seedlings level, which were germinated in stressful conditions, showed different results. Within the three analyzed provenances, two grouped within populations at lower distances (rocks-check p.). Depending on population they originated from (višegrad-rocks; višegrad-check p.), graph 5. In this case, we can identify differences in gene expression to stressful conditions, which can be attributed to differences at population level, i.e. the influence of the habitat conditons.



Graph 5. Cluster analysis of lines on the basis of the content of salt soluble proteins in seedlings in regard to half sib lines and populations

## CONCLUSIONS

The study of protein complex in seed and its reaction to drought conditions at 40 Austrian pine (*Pinus nigra Arnold*), test trees from diametrically different habitat conditions (rocks-check p.) is a complex occurrence influenced by heterogeneity and it includes different regulatory mechanisms in plant tissue. Seed analysis of the salt soluble proteins showed that genotypes have specific protein image. Protein stripes were observed in all lines, as well those which presence depend on free pollination lines or provenances. Furthermore, we identified a small number of cases of identical protein structure within the same half sib line repetitions, which speaks in favour of a high inter-linear variability, caused by father's influence and the heterozygosity of mother trees. This is proved by

variability analysis. Protein structure analysis, in seedlings grown in normal and stressful conditions, identified certain proteins to occur in all half sib lines regardless of germination conditions.

Protein complex analysis, at seed level, observed certain polypeptides to be "conservative" i.e. the presence in all free pollination lines. On the other hand, we observed protein stripes which vary at free pollination line level, and also those which presence depend on the provenance. These facts confirm partial variability of certain proteins, in other words, certain proteins in observed populations are monomorph as they occur only in one form, other loci are polymorph because provenance, population, or environmental factors (drought factors in our case) influenced their occurrence.

As long as we identify the most significant provenance differences in protein analysis of seed, we observe inter-population, population and provenance differences for 9-days-old seedlings germinated in drought and normal conditions. Depending on habitat conditions the seed originates from, the protein complex in seed can be defined as stable. Unlike this fact, its analysis at seedlings germinated in stressful conditions level, gives different results. We ascertain here the differences in gene expression to stressful conditions, which might be caused by differences at population level, i.e. habitat condition influence (at Višegrad provenance).

Qualitative, and not quantitative analysis was obtained by the analysis of salt soluble proteins which showed polypeptide's presence/absence, but not their amount. In future research work it would be interesting to monitor gene expression as a reaction to drought conditions through polypeptide amount. In order to define inter-population differences, further research activities should use DNA markers, which would precisely point out to inter-population differences.

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**PROTEINI U SEMENU I KLIJANCIMA AUSTRIJSKOG BORA  
(*PINUS NIGRA* ARNOLD) KAO GENETIČKI MARKERI  
TOLERATNOSTI NA SUŠU**

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**I z v o d**

Preduslov za stvaranje i selekciju tolerantnih genotipova na stresne uslove je proučavanje fiziološke, biohemijske i molekularne osnove njihove adaptivne reakcije na stres. Istraživanjima je obuhvaćeno 40 linija crnog bora slobodnog oprašivanja koje potiču iz 5 provenijencija: Sutjeska, Višegrad, Tara, Teslić i Durmitor (BiH, Srbije i Crna Gora). U svakoj provenijenciji izdvojene su dvije populacije i to: 5 linija slobodnog oprašivanja predstavlja populaciju sa stijena i 3 linije slobodnog oprašivanja populaciju na najboljem staništu crnog bora. Specifičnost ispitivanih provenijencija, populacija, kao i linija slobodnog oprašivanja dokazana je putem analiza u solima rastvorljivih proteina. Identičan proteinski sastav u ponavljanjima iste linije slobodnog oprašivanja je dokazan u malom broju slučajeva, što govori u prilog velike unutarlinijske varijabilnosti koja može biti posledica uticaja oca, kao i heterozigotnosti materinskih stabala. U analizama proteinskog sastava sjemena konstatovane su najznačajnije razlike na nivou provenijencija. Kod klijavaca starih 9 dana, isključili su uslovima indukovane suše i u standardnim uslovima, utvrđene su međulinijske, populacione i provenijencijske razlike, kao i značajne interakcije porekla i stresnog faktora suše.

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